

# **DEVELOPMENT AT A GLANCE**

# Semaphorin signalling during development

Bart C. Jongbloets and R. Jeroen Pasterkamp\*

# ABSTRACT

Semaphorins are secreted and membrane-associated proteins that regulate many different developmental processes, including neural circuit assembly, bone formation and angiogenesis. *Trans* and *cis* interactions between semaphorins and their multimeric receptors trigger intracellular signal transduction networks that regulate cytoskeletal dynamics and influence cell shape, differentiation, motility and survival. Here and in the accompanying poster we provide an overview of the molecular biology of semaphorin signalling within the context of specific cell and developmental processes, highlighting the mechanisms that act to fine-tune, diversify and spatiotemporally control the effects of semaphorins.

KEY WORDS: Axon guidance, Bone remodelling, Cardiac development, Cell migration, Neuropilin, Plexin, Semaphorin

Department of Translational Neuroscience, Brain Center Rudolf Magnus, UMC Utrecht, 3451 PM Utrecht, The Netherlands.

\*Author for correspondence (r.j.pasterkamp@umcutrecht.nl)

# Introduction

The development of complex tissues and organs depends on the generation, differentiation and migration of cells. Semaphorins, which constitute a large family of secreted and membrane-associated proteins, regulate many of these cell biological events in different organ systems and species. The best-characterised biological feature of semaphorins is their ability to provide repulsive or attractive cues for migrating cells and growing neurites, i.e. axons and dendrites. However, semaphorins can also influence other cellular processes, such as cell division, differentiation and survival, and hence play roles in a variety of developmental processes (Giacobini and Prevot, 2013; Kang and Kumanogoh, 2013; Kumanogoh and Kikutani, 2013; Neufeld et al., 2012; Pasterkamp, 2012; Roth et al., 2009; Tamagnone, 2012). To exert their effects, semaphorins bind multimeric receptor complexes at the cell membrane and initiate unique intracellular signal transduction cascades. An important downstream target of semaphorin signalling is the actin cytoskeleton, but semaphorin-mediated effects on other cytoskeletal proteins, or on gene and protein expression, have also been described (Cagnoni and Tamagnone, 2013; Hota and Buck, 2012; Siebold and



Jones, 2013; Tran et al., 2007; Yoshida, 2012; Zhou et al., 2008). Here, we provide an introduction to semaphorins, their receptors and their signalling mechanisms, and we highlight the different molecular mechanisms that function to spatiotemporally control or diversify the effects of semaphorins within different developmental contexts.

# The semaphorin family and its receptors

More than twenty semaphorin proteins have been identified and they are categorised into eight subfamily classes on the basis of structural and amino acid sequence similarity (Semaphorin Nomenclature Committee, 1999; Yazdani and Terman, 2006). Semaphorins found in invertebrate species are grouped into classes 1, 2 and 5 (Sema-5c); classes 3-7 contain vertebrate semaphorins (except for Sema-5c, which is found in invertebrates only); and class V contains semaphorins found in the genomes of certain DNA viruses. Semaphorin proteins are defined by a ~400 amino acid semaphorin (sema) domain, which facilitates homodimerisation and interactions with semaphorins, also contain a short, cysteine-rich plexin-semaphorin-integrin (PSI) domain. Semaphorins are further distinguished by class-specific protein domains, such as basic, immunoglobulin (Ig)-like, and thrombospondin domains.

The principal receptors for semaphorins are plexins, which are transmembrane proteins that are divided into four classes (A-D) on the basis of structural criteria (Fujisawa, 2004; Hota and Buck, 2012; Perälä et al., 2012). Nine plexins have been identified in vertebrates (plexins A1-A4, B1-B3, C1, D1) and two in invertebrates (PlexA and PlexB). The plexin extracellular region contains several different motifs and domains, including a divergent sema domain, whereas the intracellular region contains a GTPaseactivating protein (GAP) domain. Most semaphorins bind directly to plexins, but various other membrane-associated proteins can also act as receptors or co-receptors for semaphorins. For example, the binding of secreted class 3 semaphorins (except Sema3E) to plexin is facilitated by neuropilins (Fujisawa, 2004), which are type I transmembrane proteins that harbour a short cytoplasmic domain. Two neuropilins have been identified in vertebrates: neuropilin 1 (Nrp1) and Nrp2. In addition to its role as a ligand-binding co-receptor for Sema3s, Nrp1 serves as a Sema4A receptor in the immune system (Delgoffe et al., 2013). Sema7A can signal independently of plexins through  $\beta 1$  integrins, whereas Sema3A may signal through Nrp1/plexin A1 and Nrp1/L1 (L1cam) complexes (Bechara et al., 2008; Pasterkamp et al., 2003).

# Mechanisms of intracellular signalling

The signalling pathways that act downstream of semaphorin/plexin have been investigated in many different cell types and model organisms. It is important to note that, although some of these signalling pathways function downstream of plexins of different subclasses, plexin subclass-specific signalling has also been reported. In addition, plexins belonging to the same subclass may act redundantly in specific developmental processes (Schwarz et al., 2008; Yaron et al., 2005). Although a number of intracellular proteins have been identified as working with semaphorins/plexins, here we focus on three key signalling events downstream of plexins: signalling induced by the plexin GAP domain; regulation of the actin cytoskeleton; and the control of cell-cell and cell-substrate adhesion.

The binding of semaphorins to plexins leads to activation of the plexin GAP domain and subsequent signalling through downstream molecules such as protein kinases, GTPases and cytoskeletonassociated proteins (Cagnoni and Tamagnone, 2013; Hota and Buck, 2012; Tran et al., 2007; Zhou et al., 2008). Activation of the plexin GAP domain upon semaphorin binding has been shown for all classes of plexins. It reduces the levels of active Ras and Rap GTPases, and is important in vivo for semaphorin/plexin signalling (Mizumoto and Shen, 2013; Oinuma et al., 2004; Wang et al., 2012; Worzfeld et al., 2014; Yang and Terman, 2012). In vitro studies have shown that reduced levels of active Ras lead to activation of CRMP-2 (Dpysl2), a microtubule-depolymerising protein, via a PI3K-AKT-GSK3ß signalling pathway (Pasterkamp, 2005). Plexin GAP activity is not only regulated by ligand binding but also by intrinsic factors. For example, binding of the small GTPase Rnd1 to plexin B1 stimulates plexin B1 GAP activity in vitro, while PKAmediated phosphorylation of the PlexA GAP domain triggers the recruitment of 14-3-3ε, leading to reduced GAP activity in vivo (Oinuma et al., 2004; Yang and Terman, 2012).

The actin cytoskeleton is a converging target of many semaphorin signalling pathways (Hung and Terman, 2011). Studies have shown that members of the MICAL family of redox enzymes (Zhou et al., 2011) can bind to the cytosolic part of plexins and induce F-actin disassembly by mediating the post-translational oxidation of actin filament subunits (Hung et al., 2010, 2011). Intriguingly, this process is antagonised by the methionine sulfoxide reductase SelR, which reduces oxidised actin and thereby promotes F-actin assembly (Hung et al., 2013). MICALs and SelR thus provide plexin receptors with the ability to directly redox modify F-actin upon semaphorin binding. However, although Mical and SelR are required *in vivo* for *Drosophila* Sema-1a/PlexA-mediated effects during axon guidance and mechanosensory bristle development, the role of MICALs downstream of vertebrate semaphorins awaits characterisation.

Semaphorin/plexin signalling also controls cell-cell and cellsubstrate adhesion in various contexts. For example, plexins employ different molecular strategies to negatively regulate integrindependent cell adhesion. These include inactivating Ras family members, which normally act to promote integrin-mediated adhesion, in various cell types including neurons (Kinbara et al., 2003), and promoting the inhibition of PIPKIY661 by FARP2 during axon repulsion (Oinuma et al., 2006; Toyofuku et al., 2005). In addition, plexins can regulate integrin clustering and endocytosis during angiogenesis and the lymphoid trafficking of thymocytes (Choi et al., 2014; Sakurai et al., 2011; Sandri et al., 2012).

#### Spatiotemporal regulation of semaphorin expression

The expression and cell surface levels of semaphorins and their receptors are tightly controlled to regulate the magnitude, duration and spatial activity of semaphorin signalling. For example, in retinal ganglion cells (RGCs) and interneurons, the expression of Nrp1 and Nrp2 is transcriptionally repressed by the transcription factors CoREST and Nkx2-1, respectively (Baudet et al., 2011; Nobrega-Pereira et al., 2008). This reduces the responsiveness of growth cones and neurons to the repulsive Nrp ligands Sema3A and Sema3F, allowing them to invade brain regions that express these repellents (Baudet et al., 2011; Nóbrega-Pereira et al., 2008). Intriguingly, downregulation of CoREST mRNA by miR-124 releases the suppression of Nrp1 transcription by the REST/ CoREST transcriptional complex in RGCs in the eve. This triggers growth cone sensitivity to Sema3A in a spatiotemporally controlled fashion, enabling RGC growth cones to select their appropriate targets in the brain (Baudet et al., 2011). Transcripts encoding semaphorins and their receptors can also be direct targets of miRNAs (Baudet et al., 2013; Lee et al., 2012; Urbich et al., 2012).

In addition to miRNAs, Nrp levels are controlled by the cyclic nucleotide cAMP. In both the developing zebrafish optic chiasm and the mouse olfactory system, cAMP promotes Nrp expression and thereby sensitivity to Sema3 proteins during axon guidance (Dell et al., 2013; Imai et al., 2006).

In addition to these more global modes of expression regulation, the expression of semaphorins and their signalling molecules is controlled locally in specific subcellular compartments. For example, in neuronal growth cones, the binding of Sema3A to its receptor complex promotes the local synthesis of its own downstream signalling molecules, including RhoA and ADF/ cofilin (Campbell and Holt, 2001; Manns et al., 2012; Piper et al., 2005; Wu et al., 2005). Furthermore, endocytosis, receptor phosphorylation, membrane protein shedding, intracellular protein degradation and protein transport have been identified as strategies to control semaphorin signalling at specific times and places in the cell (e.g. Dang et al., 2012; Nawabi et al., 2010; Yang and Terman, 2012; Zhu et al., 2007).

# **Diversification of semaphorin signalling**

Our organs consist of millions of cells but only a limited set of molecular cues is available to regulate cell migration, differentiation and survival. Accumulating evidence indicates that distinct molecular mechanisms act to diversify the effects of semaphorins, and other cues, allowing them to control a disproportionally large number of different cellular events. Below, we discuss examples of important semaphorin signalling and diversification strategies within the context of different cell and developmental processes that showcase these mechanisms.

#### **Reverse signalling**

Most of the biological effects exerted by semaphorins rely on semaphorin forward signalling, i.e. signalling downstream of plexins following semaphorin ligand binding. However, semaphorins serve not only as ligands but also in some instances as receptors. For example, in the embryonic chick heart, Sema6D acts both as a ligand and as a receptor for plexin A1 (Toyofuku et al., 2004a,b). During the process of ventricular trabeculation, proliferating cells residing in the compact myocardial cell layer generate myocardial cells that migrate out into the overlaying trabecular myocardium. Studies have shown that interactions between myocardial cells expressing Sema6D and plexin A1 in the compact layer induce forward and reverse signalling to induce circumferential migration of these cells, leading to expansion of the compact layer. The migration of myocardial cells out of the compact layer requires Sema6D-plexin A1 reverse signalling; myocardial cells migrating towards the trabecular layer express Sema6D but not plexin A1, and the interaction of these cells with those expressing Sema6D and plexin A1 in the compact layer triggers reverse signalling, causing their repulsion into the trabecular layer (Toyofuku et al., 2004b). It was shown that the binding of plexin A1 to Sema6D triggers the recruitment of activated Abl to the Sema6D-expressing cytoplasmic region, leading to phosphorylation of the mammalian cytoskeletal regulator enabled (Toyofuku et al., 2004b). Upon entry into the trabecular layer, myocardial cells induce Sema6D forward signalling to position endocardial cells. Sema6D released from myocardial cells into the cardiac jelly suppresses the inward migration of plexin A1-positive endocardial cells covering the trabeculae (Toyofuku et al., 2004b).

Reverse signalling has also been observed during neural circuit assembly in *Drosophila*. Sema-1a, the invertebrate semaphorin most closely related to Sema6 proteins, can serve as a receptor in synaptogenesis and during the targeting of axons and dendrites in *Drosophila* (Cafferty et al., 2006; Godenschwege et al., 2002; Jeong et al., 2012; Komiyama et al., 2007; Yu et al., 2010). Downstream of Sema-1a, RhoGAPp190 and Pebble, a Rho-GEF, function to regulate Rho activity and control motor axon fasciculation and target recognition (Jeong et al., 2012). The ability of such membrane-associated semaphorins to interact with signalling proteins through their cytoplasmic domains suggests that many other semaphorins might be capable of mediating reverse signalling events.

# Cis inhibition and activation

In addition to being expressed in separate cells and acting in *trans*, semaphorins and plexins also engage in *cis* interactions, and three different modes of such interactions have been described.

First, the binding of a semaphorin to its plexin receptor in *cis* can prevent interactions between this plexin and semaphorin ligands presented in *trans*. This mode of inhibitory receptor regulation has been shown for Sema6A and its receptor plexin A4 in mouse dorsal root ganglion (DRG) and sympathetic neurons. Both of these neuronal cell types express plexin A4, but only the growth cones of sympathetic neurons display strong repulsive responses to Sema6A; the binding of Sema6A to plexin A4 in *cis* in DRG neurons hinders *trans* interactions between Sema6A and plexin A4, thereby reducing Sema6A responsiveness *in vitro* (Haklai-Topper et al., 2010). Similarly, Sema6A binds plexin A2 in *cis* in a subset of starburst amacrine cells (SACs) in the mouse retina to control Sema6A repulsive responses *in vitro* and SAC stratification and morphology *in vivo* (Sun et al., 2013).

Second, the binding of a plexin receptor to its ligand in *cis* can block interactions between this ligand and plexin receptors expressed on adjacent cells or axons. This mode of interaction is evident during the development of the hippocampus (Suto et al., 2007). Axon projections from granule cell neurons in the dentate gyrus, known as mossy fibres, express plexin A4, whereas the repulsive plexin A4 ligand Sema6A is expressed in the target area of these axons, the CA3 region. Although Sema6A is present throughout the CA3 region, the binding of a different plexin – plexin A2 – to Sema6A in *cis* renders Sema6A unavailable for *trans* interactions with plexin A4 on mossy fibre axons in a specific layer of the target. Plexin A2-Sema6A interactions thereby create a non-repulsive corridor that is invaded by mossy fibre axons leading to a layer-restricted innervation pattern (Suto et al., 2007).

Third, *cis* interactions between semaphorins and plexins can activate plexin signalling *in cis*. Such *cis* interactions have been documented between the transmembrane semaphorin SMP-1 and the class A plexin homologue PLX-1 in *C. elegans* motoneuron axons. This interaction induces signalling downstream of PLX-1, leading to the inhibition of synapse formation (Mizumoto and Shen, 2013).

#### Modulatory co-receptors

Co-receptor proteins provide plexin-containing receptor complexes with unique signalling capacities, and the presence of specific coreceptors often determines the cellular response to a particular semaphorin (e.g. Castellani et al., 2000; Falk et al., 2005; Kantor et al., 2004; Swiercz et al., 2004). This is exemplified by the role of Sema3E during the formation of long axon tracts in the brain. Axons from ventrolateral cortical and striatal neurons express the Sema3E receptor plexin D1 and display repulsive responses to Sema3E. Sema3E expression thus dictates repulsive territories that prevent striatal and cortical axons from entering inappropriate regions of the brain. By contrast, subicular axons express Nrp1 and VEGFR2 (Kdr) in addition to plexin D1, and the inclusion of Nrp1 and VEGFR2 into the receptor complex switches the response to Sema3E from repulsion to attraction. Subicular axons are therefore attracted by Sema3E, which is secreted by adjacent axons and serves as an attractive scaffold for navigating subicular axons (Chauvet et al., 2007). Intriguingly, Sema3E does not bind Nrp1 or VEGFR2, but rather uses VEGFR2 as a signal-transducing receptor subunit following binding to plexin D1. This is in contrast to signalling during Sema3E-mediated repulsion, which depends on plexin D1 both as a ligand-binding and a signal-transducing receptor (Bellon et al., 2010). For most of the semaphorin co-receptor proteins identified to date, it remains to be determined how they influence plexin signalling. Roles in ligand presentation, recruitment of additional signalling molecules and modulation of downstream signalling events are likely, based on what is known about other multisubunit receptors with related functions.

# **Competitive ligand interactions**

Interactions between semaphorins and their receptors are exquisitely complex. For example, semaphorins belonging to different classes can bind and signal through members of the same plexin subclass. Likewise, homologous semaphorins, such as Sema-2a and Sema-2b, can signal through the same plexin, PlexB, but elicit opposing effects (Wu et al., 2011). Semaphorins interacting with the same plexin may also compete for binding. This mode of competitive ligand interactions has been most thoroughly characterised during bone remodelling, but most likely also functions in other organ systems (Havashi et al., 2012; Takahashi et al., 1998). Bone homeostasis depends on a balance between bone formation and resorption mediated by osteoblasts and osteoclasts, respectively. The binding of Sema6D to plexin A1, which is expressed on pre-mature osteoclasts in complex with the receptor proteins Trem2 and DAP12 (Tyrobp), triggers the differentiation of bone-resorbing osteoclasts (Havashi et al., 2012). Sema3A, which is released by sensory afferents innervating the bone (Fukuda et al., 2013), antagonises the effects of Sema6D by sequestering plexin A1 away from the Trem2/DAP12 complex. In addition, the binding of Sema3A to the plexin A1/Nrp1 receptor complex triggers the differentiation of bone-forming osteoblasts and inhibits osteoclast precursor cell migration. Collectively, these effects of Sema3A regulate bone formation.

#### Semaphorins as semaphorin receptors

Recent work in the developing Drosophila olfactory system has raised the possibility that the functions of certain semaphorins might be mediated by other transmembrane semaphorins acting as receptors. The development of functional olfactory circuits in Drosophila requires the proper targeting of projection neuron (PN) dendrites to specific regions in the antennal lobe (AL). The AL is a highly organised structure in which axons from different classes of olfactory receptor neurons (ORNs) contact specific groups of PN dendrites in dedicated areas called glomeruli. During development, larval ORN axons, which secrete Sema-2a and Sema-2b, project to the AL. These axons slowly degenerate as the fly develops, thus generating a temporally receding gradient of Sema-2 in the AL. The expression of Sema-1a, but not of plexins, in PNs is required to sense this repulsive Sema-2 gradient; PN dendrites expressing high levels of Sema-1a are directed to the dorsolateral AL, whereas weak Sema-1a expression allows ventromedial PN dendrites to extend towards the ventromedial AL (Sweeney et al., 2011). This pre-patterning of PN dendrites by degenerating larval ORN axons ensures the proper subsequent innervation of the AL by adult ORN axons. Together, these results

support the intriguing idea that Sema-1a can act as a (co)receptor for Sema-2 proteins.

# **Conclusions and perspectives**

Work in recent decades has unveiled the remarkably pleiotropic nature of semaphorins during development. Recent studies focusing on the molecular basis of semaphorin signalling have uncovered molecular strategies that allow a limited set of semaphorins to influence a disproportionally large number of cells in a highly spatiotemporally controlled fashion. These studies have, for example, highlighted the existence of inhibitory and stimulatory cis interactions between semaphorins and plexins, and the ability of transmembrane semaphorins to function as receptors. However, many intriguing questions remain to be addressed. For example, how are the different modes of interaction between plexins and semaphorins regulated? Do the mechanisms identified for individual semaphorins (e.g. transmembrane semaphorins serving as receptors for other semaphorins) apply more generally in different organ systems and species? As cells migrate and axons extend they not only encounter semaphorins but may simultaneously detect other chemotropic cues. How are these different signals integrated, and where and how do semaphorin signalling pathways intersect with other pathways? Answers to these and other questions will not only further our understanding of semaphorin function and signalling during development, but will also help to address the roles of these cues in the adult organism and during disease.

# Acknowledgements

We thank Jonathan Terman, Atsushi Kumanogoh and Alex Kolodkin for critical reading of the manuscript and apologise to the many colleagues whose work could not be cited owing to space limitations.

#### **Competing interests**

The authors declare no competing financial interests.

#### Funding

Research on semaphorins in the laboratory of the authors is supported by the Netherlands Organization for Health Research and Development (ZonMW-TOP), UMC Utrecht and the National Epilepsy Foundation [NEF 10-17] (to R.J.P.) and is partly performed within the framework of the Center for Translational Molecular Medicine (CTMM), project EMINENCE [01C-204] (R.J.P.).

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